

TITLE: Methods of Using Agents that Modulate Bone Formation and Inhibit Adipogenesis

INVENTORS: Roland E. Baron, Natalie Sims, Geogios Sabatakos, Eric Nestler, Jingshan Chen, and Max Kelz

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application 60/228,450, filed August 29, 2000, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to methods of identifying agents that modulate Δ FosB expression. Specifically, the present invention relates to identifying agents that modulate osteogenesis and adipogenesis. The present invention also relates to methods of identifying genes modulated by Δ FosB or modulates Δ FosB expression and associated with osteogenesis and adipogenesis. Additionally, the present invention relates to using the identified agents to treat subjects with diseases or conditions associated with abnormal bone formation and abnormal adipogenesis.

BACKGROUND:

Bone Formation

[0003] Bone formation, *i.e.* osteogenesis, is essential for the maintenance of bone mass in the adult skeleton. It begins during prenatal development and persists throughout adulthood. There are two ways in which osteogenesis occurs: intramembranous ossification and endochondral ossification.

[0004] There are two types of cells that are important in osteogenesis. Osteoblasts, involved in both endochondral and intramembranous ossification, are the specialized cells in bone tissue that make matrix proteins resulting in the formation of new bone. These

bone-forming cells are derived from mesenchymal osteoprogenitor cells. They form an osseous matrix in which they become enclosed as an osteocyte. They are capable of differentiating to other lineages such as adipocytes, chondrocytes and muscle (Bellows et al., 1994). Unlike osteoblasts, osteoclasts are used in endochondral ossification. They dissolve calcium previously stored away in bone and carry it to tissues whenever needed. Thus, while osteoblasts are associated with new bone growth, osteoclasts are associated with bone absorption and removal.

[0005] Bone is subject to constant breakdown and resynthesis in a complex process mediated by osteoblasts, which produce new bone, and osteoclasts, which destroy bones. In normal bone, the balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption is maintained through complex regulating interactions.

Conditions and Diseases Associated with Bone Formation

[0006] There are many deficiencies, diseases, and disorders associated with the skeletal system. Examples of a few include, but are not limited to, osteoporosis, bone cancer, arthritis, rickets, bone fracture, periodontal disease, bone segmental defects, osteolytic bone disease, primary and secondary hyperparathyroidism, Paget's disease, osteomalacia, hyperostosis, and osteopetrosis.

[0007] Accordingly, there is a need to develop methods of treating diseases associated with bone growth disorders, methods of hastening bone formation, methods of identifying agents that modulate (increase or decrease) bone formation, and methods of identifying genes associated with bone growth disorders.

Δ FosB

[0008] The AP-1 family of transcription factors consists of dimeric complexes of Fos-related (c-Fos, FosB, Δ FosB, Fra-1 and Fra-2) and Jun-related (cJun, JunB and JunD) proteins. These are basic leucine zipper proteins that modulate the transcription of a

variety of genes via interactions with specific sequences on the promoters of target genes. Members of the AP-1 family participate in the regulation of bone cell proliferation and differentiation, and the promoters of several genes involved in bone formation contain AP-1 consensus sequences. In addition, several regulators of bone formation induce AP-1 expression in early osteoblast precursors (Grigoriades *et al.*, 1993). Furthermore, mice overexpressing c-Fos develop osteosarcomas (Grigoriades *et al.*, 1993, Wang *et al.*, 1992) while c-*fos* knockout mice lack osteoclasts and develop osteopetrosis (Johnson *et al.*, 1992; Wang *et al.*, 1992). In contrast, no bone abnormalities have been described in *fosB* knockout mice (Gruda *et al.*, 1996), nor in mice where FosB, Fra-2, c-Jun or JunB alone were overexpressed (Grigoriadis *et al.*, 1993).

[0009] Despite numerous studies on the role of AP-1 family members in skeletal biology, the role of Δ FosB, a Fos-related protein that arises from alternative splicing of the *fosB* transcript, has never been analyzed. Δ FosB can be induced in a region-specific manner in brain in response to several types of chronic perturbation, including drugs of abuse, antipsychotic drugs, antidepressant drugs, seizures and lesions (Chen *et al.*, 1998). Once induced, Δ FosB isoforms persist in brain for relatively long periods due to their extraordinary stability. Mice lacking the *fosB* gene show abnormal biochemical and behavioral responses to chronic administration of drugs of abuse or antidepressant treatments, consistent with an important role for Δ FosB in mediating long-term adaptations in the brain (Nestler *et al.*, 1999).

The Relationship Between Adipogenesis and Osteogenesis

[0010] Adipocytes form an integral part of the stromal system of bone and marrow and participate in the establishment and maintenance of the hematopoietic microenvironment (Beresford *et al.*, 1992). Adipocytic and osteogenic cells are the two main cell lines of the marrow stromal system. There is also evidence that they are derived from a multipotential stromal stem cell in the adult marrow (Beresford *et al.*, 1992). Beresford *et al.* (1992) report that there is evidence for an inverse relationship between the expression

of the adipocytic and osteogenic phenotypes in cultures of rat marrow stromal cells. Beresford *et al.* (1992) also report that this finding is consistent with the possibility that the regulation of adipogenesis and osteogenesis can occur at the level of a common precursor.

[0011] Gimble *et al.* (1995) describe the effects of bone morphogenetic proteins on adipogenesis in a multipotent murine bone marrow stromal cell line, BMS2. Gimble *et al.* (1995) report that bone morphogenetic proteins inhibit adipocyte differentiation in multipotent bone marrow stromal cells *in vitro*.

Diseases Associated with Adipogenesis

[0012] Diseases associated with adipogenesis include body weight disorders such as obesity, anorexia, cachexia, and nonshivering and shivering thermogenesis. Obesity is usually defined as body weight of more than 20% in excess of the ideal body weight. Obesity is associated with an increased risk for cardiovascular disease, diabetes, and an increased mortality rate (Grundy *et al.*, 1990, Disease-a-Month 36:645-696). On the other hand, loss of appetite, diminished food intake, and loss of body weight are also problems associated with many diseases.

[0013] Thus, there is also a need to develop methods of treating diseases associated with adipogenesis, methods of identifying agents that modulate adipogenesis, and methods of identifying genes associated with adipogenesis.

SUMMARY OF THE INVENTION

[0014] The present invention provides a method of identifying an agent that modulates bone formation or osteogenesis. Preferably, this method is practiced by administration of a test agent, and monitoring expression of Δ FosB to determine whether the agent modulates bone formation, wherein an increase in the expression of Δ FosB indicates that the agent is an inducer of bone formation and wherein a decrease in the expression of Δ FosB indicates that the agent is an inhibitor of bone formation.

[0015] The present invention also provides a method of identifying an agent that interacts with Δ FosB comprising incubating a test agent with Δ FosB and detecting an interaction between Δ FosB and the test agent.

[0016] In one embodiment of the invention, the agent is administered to isolated cells in culture, such as, but not limited to, osteoblasts and chondrocytes, preferably, primary osteoblasts, MC3T3-E1 cells, or C2C12 cells. In another embodiment, the agent is administered to cell lysates, specifically nuclear extracts. In a third embodiment of the invention, the agent is administered to a non-human transgenic animal, preferably mice and most preferably mice that overexpress Δ FosB.

[0017] The present invention also provides a method of identifying an agent that modulates adipogenesis. Preferably, this method is practiced by administration of a test agent and monitoring expression of Δ FosB to determine whether the agent modulates adipogenesis, wherein an increase in the expression of Δ FosB indicates that the agent is an inhibitor of adipogenesis and wherein a decrease in the expression of Δ FosB indicates that the agent is an inducer of adipogenesis.

[0018] In one embodiment of the invention, the agent is administered to isolated cells in culture, such as, but not limited to, primary adipocytes, 3T3-L1 preadipocytes, 3T3 F422A, and ob 1771. In another embodiment, the agent is administered to cell lysates, specifically nuclear extracts. In a third embodiment of the invention, the agent is administered to a non-human transgenic animal, preferably mice and most preferably mice that overexpress Δ FosB.

[0019] Alternatively, the methods of the present invention may be practiced using a yeast two hybrid system or reporter gene system to monitor the expression of Δ FosB.

[0020] Moreover, the present invention provides a method of modulating the differentiation or proliferation of cells including but not limited to osteoblast, bone marrow stromal cells, or pluripotent precursor cells by administering an agent that modulates bone formation or Δ FosB expression.

[0021] In one aspect, the present invention contemplates a method of modulating differentiation or proliferation of cells including but not limited to adipocytes, preadipocytes, bone marrow stromal cells, or pluripotent precursor cells by administering an agent that modulates adipogenesis or Δ FosB expression.

[0022] In another aspect, the present invention contemplates using agents identified to modulate bone formation or adipogenesis for treating diseases associated with osteogenesis or adipogenesis. These diseases include but are not limited to osteoporosis, bone cancer, arthritis, rickets, bone fracture, periodontal disease, bone segmental defects, osteolytic bone disease, primary and secondary hyperparathyroidism, Paget's disease, osteomalacia, hyperostosis, osteopetrosis, osteosclerosis, osteoporosis, obesity, anorexia, cachexia, and nonshivering and shivering thermogenesis.

[0023] Further, the present invention provides a method of identifying genes that are modulated by Δ FosB comprising inducing Δ FosB in a cell and determining which genes are differentially expressed, thereby identifying genes that are modulated by Δ FosB or associated with Δ fosB mediating its action on bone formation or adipogenesis.

Preferably, this method is performed using a yeast two-hybrid system or hybridization of cellular nucleic acids to a DNA chip, for example, to be used in target identification.

[0024] The present invention also provides a method of identifying genes that modulate Δ FosB expression comprising measuring the expression level of Δ FosB in the presence of test genes, thereby identifying genes that modulate Δ FosB. The test genes may originate from a nucleic acid library and the test genes may be present on a heterologous vector. The expression level of Δ FosB may be determined by using Northern blot analysis, Western blot analysis, PCR analysis, or two hybrid screening assays, or a reporter gene system. The contemplated reporter gene system may comprise a reporter gene linked to a promoter that interacts with Δ FosB. Additionally, the method comprises Δ FosB linked to a heterologous protein. The method may be performed using a cell based or cell free system. Further, the method may be performed using high throughput assays using, for example, DNA chip.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] Figures 1 A-C. Increased bone density and tissue-specific expression of Δ FosB isoforms in the NSE- Δ FosB mice.

[0026] A. Contact radiograph of 15 week old control (left) and 1A Δ FosB littermates (right). B. Schematic diagram of the functional domains of FosB and Δ FosB (upper panel): FHD: Fos-homology domain, BR: basic region, LZ: leucine zipper, PPP: proline-rich transactivation domain. *In vitro* transcription/translation of Δ FosB (Δ) or of mutants lacking the first (Δ 1) or the first and second (Δ 2) methionine residues (bottom panel) (Chen *et al.*, 1997). C. Tissue-specific expression of Δ FosB isoforms in adult control (C) and 11A (Δ) mice (left panel): muscle (Mus), bone, skin, brain, lung, heart, adipose (Adip) and spleen (Spl). Δ FosB isoforms are expressed in osteoblasts derived from calvariae of control (C) and Δ FosB (Δ) animals (right panel).

[0027] Figures 2 A-C. Progressive osteosclerosis in mice overexpressing Δ FosB.

[0028] A. Von Kossa-stained proximal tibiae from control (lower panel) and 1A Δ FosB (upper panel) mice at 6 days (d6), 4 weeks (4wk), 8 weeks (8wk), 10 weeks (10wk) and 15 weeks (15wk). B. Bone formation: Increased trabecular bone volume (BV/TV), bone formation rate (BFR/BV), mineral appositional rate (MAR), osteoblast surface (ObS/BS), osteoblast number (ObN/BPm) and serum osteocalcin (OCN) in Δ FosB expressing mice (11A and 1A) and controls (Ctrl) at 10 weeks of age. Northern blot analysis of total RNA from calvariae of control (C) and 11A Δ FosB (Δ) mice at 2 days postnatally, demonstrating higher osteocalcin (OC), type I collagen (Col I) and osteopontin (OP) mRNA expression in Δ FosB mice. C. Bone resorption: Unchanged osteoclast surface (OcS/BS), and urinary deoxypyridinoline crosslinks (DPyr/Cr) in 10 week old control (Ctrl) and Δ FosB (11A) mice. Number of tartrate resistant acid phosphatase stained multinuclear cells (TRAP+ MNCs) detected in *in vitro* osteoclastogenesis analysis of primary osteoblasts (OB) and bone marrow cells (BM) from control (Ctrl) and Δ FosB

(11A) mice. Bone resorption of authentic osteoclasts from control (Ctrl) and Δ FosB (11A) mice on dentin slices.

[0029] Figures 3 A-D. Cell-autonomous increased osteoblastogenesis in primary calvarial cultures of Δ FosB mice.

[0030] A. Extracellular matrix mineralization in control (C) and Δ FosB (Δ) calvarial cultures at days 6 (d6) and 14 (d14), Von Kossa staining. B. Quantitation of mineralization in control (open bars) and Δ FosB (shaded bars) cultures at days 6 (d6), 14 (d14), 21 (d21) and 31 (d31). C. Northern blot analysis of total RNA of control (C) and Δ FosB (Δ) primary osteoblast cultures at confluency (d0). Col I: type I collagen, OP: osteopontin. Equal loading was verified by ethidium bromide staining. D. Western blot analysis of whole cell extracts from control (C) and Δ FosB (Δ) primary osteoblast cultures at confluency (d0). Equal protein loading was demonstrated by reprobing with an anti-actin antibody.

[0031] Figures 4 A-E. Decreased adipogenesis in primary calvarial and bone marrow stromal cell cultures of Δ FosB mice.

[0032] A. Adipocyte numbers in control (open bars) and Δ FosB (shaded bars) osteoblast cultures. B. Alkaline phosphatase / Oil Red O-staining of primary Δ FosB (top panel) and control (bottom panel) calvarial cultures at day 14 of mineralization. C. Western blot analysis of whole cell extracts from primary control (C) and Δ FosB (Δ) calvarial cultures at days 4 (d4) and 14 (d14) of mineralization. D. Abdominal fat weight in 10 week old control (C) and Δ FosB (1A and 11A) mice. E. Northern blot analysis of total RNA from primary control (C) and Δ FosB (Δ) bone marrow stromal cell cultures, treated with adipogenic agents as described in Methods, OP: osteopontin, Col I: type I collagen. Equal loading was verified by ethidium bromide staining.

[0033] Figures 5 A-F. Molecular *in vitro* analysis of the role of Δ FosB isoforms during osteoblast differentiation.

[0034] A. Nuclear extracts of primary confluent (d0) and mineralizing cultures from calvariae of CD1 mice at days 4 (D4), 14 (D14) and 21 (D21) were analyzed for the expression of FosB and Δ FosB isoforms by Western blot. B. Northern blot analysis of total RNA from C2C12 cells stably transfected with empty vector (C), Δ FosB (Δ) and Δ 2 Δ FosB (Δ 2) isoforms. Cells were treated with 300ng/ml rhBMP-2 for 48hrs prior to harvesting for RNA extraction. OC: osteocalcin. Equal loading is demonstrated by ethidium bromide staining. C. Western blot analysis of nuclear extracts following transient transfection of primary osteoblasts derived from CD1 calvariae with empty vector (C), Δ FosB (Δ) or Δ 2 Δ FosB (Δ 2). D. Northern blot analysis of total RNA from 3T3-L1 pre-adipocytes, stably transfected with empty vector (C), Δ FosB (Δ) and Δ 2 Δ FosB (Δ 2). Cells were either untreated (UN) or stimulated with insulin, isobutylmethylxanthine and dexamethasone (INS/IBMX/DEX) for 48 hours prior to RNA extraction. Equal loading was verified by ethidium bromide staining. E. Nuclear extracts from primary osteoblast cultures of control (C) and Δ FosB mice (left panel) and from the C2C12 stable cell lines expressing empty vector (C), Δ FosB (Δ), and Δ 2 Δ FosB (Δ 2) [right panel] were analyzed by EMSA using a consensus AP-1 oligonucleotide probe. Complexes formed are indicated by arrows. F: reaction mixture containing radiolabelled AP-1 probe without nuclear extracts. F. Antibody-supershift analysis of the complexes formed on a consensus AP-1 oligonucleotide probe using extracts from the C2C12 stable cell lines overexpressing the Δ FosB (Δ) and Δ 2 Δ FosB (Δ 2) isoforms. Antibodies used are indicated; supershifted complexes are depicted by arrows.

DETAILED DESCRIPTION OF THE INVENTION

A. General Description

[0035] The present invention is based on the finding that overexpression of Δ FosB in mice, using a tetracycline-regulated system, resulted in increased bone formation

throughout the skeleton and profound osteosclerosis. This phenotype is cell-autonomous in osteoblasts. In addition, Δ FosB inhibits adipogenesis both *in vivo* and *in vitro*, and downregulates the expression of early markers of adipocyte differentiation.

[0036] The present invention provides methods of identifying agents that modulate osteogenesis and adipogenesis by monitoring the expression of Δ FosB. The present invention also contemplates the use of the identified agents to treat specific diseases associated osteogenesis or adipogenesis. Moreover, the present invention provides methods of identifying genes that are modulated by Δ FosB or modulates Δ FosB. These genes are useful as tools for diagnosing and treating diseases, and as targets for identifying potential drugs for treating diseases.

B. Specific Embodiments

1. Δ FosB

[0037] Δ FosB is derived from the *fosB* gene via alternative splicing. As used herein the term " Δ FosB" refers to a protein described by Nakabeppu *et al.* (1991, which is incorporated herein by reference in its entirety). The term also encompasses naturally occurring Δ FosB from various animal species and allelic variants that have a slightly different amino acid sequence than the protein described by Nakabeppu *et al.* (1991). Contemplated Δ FosB from other species include but are not limited to human, rabbit, rat, murine, porcine, bovine, ovine, equine, and non-human primate species. Allelic variants, though possessing a slightly different amino acid sequence, will still have the ability to modulate bone formation.

[0038] Moreover, the term includes conservative variants of Δ FosB described by Nakabeppu *et al.* (1991). Conservative variant refers to alterations in the amino acid sequence of Δ FosB that do not alter the functional activity of Δ FosB such as modulating bone formation. The amino acid sequence of Δ FosB can be altered, for example to render Δ FosB more hydrophilic or hydrophobic without adversely affecting the activity of Δ FosB.

[0039] Such conservative mutations include but are not limited to mutations that switch one amino acid for another within one of the following groups:

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly;
2. Polar, negatively charged residues and their amides: Asp, Asn, Glu and Gln;
3. Polar, positively charged residues: His, Arg and Lys;
4. Large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and
5. Aromatic residues: Phe, Tyr and Trp.

The types of substitutions selected may be based on the analysis of the frequencies of amino acid substitutions between homologous proteins of different species developed by Schulz *et al.*, Principles of Protein Structure, Springer-Verlag, 1978, pp. 14-16, on the analyses of structure-forming potentials developed by Chou and Fasman, Biochemistry 13, 211, 1974 or other such methods reviewed by Schulz *et al.*, Principles in Protein Structure, Springer-Verlag, 1978, pp. 108-130, and on the analysis of hydrophobicity patterns in proteins developed by Kyte and Doolittle, J. Mol. Biol. 157: 105-132, 1982.

[0040] The term "ΔFosB" also includes peptides that retain the functional activity of ΔFosB such as modulating bone formation. The peptides can be any length of at least 5, 8, 10, 15, 20 or more amino acids. It is also pointed out that these peptides are useful as antigens for generating antibodies.

[0041] Further, the term encompasses protein isolated from a natural source. A protein is said to be isolated when physical, mechanical, or chemical methods are employed to remove the protein from cellular constituents that are normally associated with the protein. A skilled artisan can readily employ standard purification methods to obtain an isolated ΔFosB protein. The term also encompasses ΔFosB protein obtained by recombinant means or chemical means.

[0042] As used herein, the term "nucleic acid encoding ΔFosB" encompasses deoxyribonucleic acids (DNAs) and ribonucleic acids (RNAs) including but not limited to

genomic DNA, cDNA, mRNA, and antisense molecules. The nucleic acid molecules of the present invention also include native or synthetic, RNA, DNA, or cDNA, that encode Δ FosB. The term includes nucleic acids encoding all of the Δ FosB proteins encompassed by the term " Δ FosB" discussed above, including but not limited to, variants, allelic variants, and naturally occurring Δ FosB from various animal species.

2. Transgenic Animals

[0043] As used herein, the term "transgenic animal" refers to any animal, preferably a non-human animal, whose genome has been altered by the introduction of a transgene. The "non-human animals" of the invention include vertebrates such as rodents, non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, *etc.* Preferred non-human animals are selected from the rodent family including rat and mouse, most preferably mouse.

[0044] As used herein, the term "transgene" refers to a nucleic acid sequence (encoding, *e.g.*, a IL-6 polypeptide), which is partly or entirely heterologous, *i.e.*, foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (*e.g.*, it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can include one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal expression of a selected nucleic acid.

[0045] The procedure for producing a transgenic animal is known in the art (Hogan *et al.*, (1986) "Manipulating the Mouse Embryo: A Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA; and U.S. Pat. No. 4,873,191). Generally, the transgene is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation

refers to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. The transgene encoding a desired polypeptide, for example Δ FosB, is linked to one or more regulatory regions. Selection of the appropriate regulatory region or regions is a routine matter, within the level of ordinary skill in the art. The regulatory regions may comprise a promoter region for functional transcription, as well as a region situated 3' of the gene of interest, and which specifies a signal for termination of transcription and a polyadenylation site. It may also include an enhancer region.

[0046] Promoters that may be used in the present invention include both constitutive promoters and regulated (inducible) promoters. The promoter may be naturally responsible for the expression of the nucleic acid. It may also be from a heterologous source. In particular, it may be promoter sequences of eukaryotic or viral genes. For example, it may be promoter sequences derived from the genome of the cell which it is desired to infect. Likewise, it may be promoter sequences derived from the genome of a virus, including the adenovirus used. In this regard, the promoters include but are not limited to ELA, MLP, HCMV and RSV genes and the like. In addition, the promoter may be modified by addition of activating or regulatory sequences or sequences allowing a tissue-specific or predominant expression.

[0047] To control the expression of a transgene product, the transgene is linked to an inducible promoter. Inducible promoters include any promoter capable of increasing the amount of gene product produced, by a given gene, in response to exposure to an inducer. Inducible promoters are known to those familiar with the art and a variety exist that could conceivably be used to drive expression of the transgene. Examples of some inducible promoters include mouse mammary tumor virus (MMTV) steroid-inducible promoter, heat shock promoter, HLA-DR promoter, steroid hormone receptor (Cicatiello *et al.*, 1995, Mol Endocrinol, 9: 1077-1090), retinoic acid receptor (Mendelsohn *et al.*, 1994, Dev (1994) 45(3): 227-241), tetracycline-regulated transcriptional modulators (Furth *et al.*, 1994, Proc Natl Acad Sci., 91(20): 9302-9306.), cytomegalovirus (CMV) immediate-

early; retroviral LTR (Choate *et al.*, 1997 Hum Gene Ther., 8(8):895-901.), metallothionein-1 (Fattori *et al.*, 1994, Blood 83:2570-2579). Tetracycline-regulated transcriptional modulators and CMV promoters are also described in WO 96/01313, and US Patent Nos. 5,168,062, and 5,385,839.

[0048] The most widely used method for the production of transgenic animals is the microinjection of DNA into the pronuclei of fertilized embryos. This method is efficient for the production of transgenic mice but is much less efficient for the production of transgenic animals using large mammals such as cows and sheep. For example, it has been reported that 1,000 to 2,000 bovine embryos at the pronuclear stage must be microinjected to produce a single transgenic cow at an estimate cost of more than \$500,000 (Wall *et al.*, (1992) J. Cell. Biochem. 49:113). Furthermore, microinjection of pronuclei is more difficult when embryos from domestic livestock (*e.g.*, cattle, sheep, pigs) is employed as the pronuclei are often obscured by yolk material. While techniques for the visualization of the pronuclei are known (*i.e.*, centrifugation of the embryo to sediment the yolk), the injection of pronuclei is an invasive technique which requires a high degree of operator skill.

[0049] Alternative methods for the production include the infection of embryos with retroviruses or with retroviral vectors. Infection of both pre- and post-implantation mouse embryos with either wild-type or recombinant retroviruses has been reported (Jaenisch (1976) Proc. Natl. Acad. Sci. USA, 73:1260-1264; Jaenisch *et al.* (1981) Cell 24:519; Stuhlmann *et al.*, (1984) Proc. Natl. Acad. Sci. USA, 81:7151; Jahner *et al.*, (1985) Proc. Natl. Acad. Sci. USA, 82:6927-6931; Van der Putten *et al.*, (1985) Proc. Natl. Acad. Sci. USA, 82:6148-6152; Stewart, *et al.*, (1987) EMBO J., 6:383-388). The resulting transgenic animals are typically mosaic for the transgene since incorporation occurs only in a subset of cells which form the transgenic animal. In addition to the production of mosaic founder animals, infection of embryos with retrovirus (which is typically performed using embryos at the 8 cell stage or later) often results in the production of founder animals containing multiple copies of the retroviral provirus at

different positions in the genome which generally will segregate in the offspring.

Infection of early mouse embryos by co-culturing early embryos with cells producing retroviruses requires enzymatic treatment to remove the zona pellucida (Hogan *et al.* (1994) in *Manipulating the Mouse Embryo: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp. 251-252).

[0050] An alternative means for infecting embryos with retroviruses is the injection of virus or virus-producing cells into the blastocoele of mouse embryos (Jahner *et al.*, 1982) *Nature* 298:623-628). As is the case for infection of eight cell stage embryos, most of the founders produced by injection into the blastocoele will be mosaic. The introduction of transgenes into the germline of mice has been reported using intrauterine retroviral infection of the midgestation mouse embryo (Jahner *et al.*, 1982 *supra*).

[0051] In summary, a method for producing a transgenic animal, which can be stably bred to produce offspring containing the gene, comprises the following steps:

- (a) isolating a fertilized oocyte from a first female animal;
- (b) transferring the transgene into the fertilized oocyte;
- (c) transferring the fertilized oocyte containing the transgene to the uterus of the same species as the first animal;
- (d) maintaining the second female animal such that
 - (i) the second female animal becomes pregnant with the embryo derived from the fertilized oocyte containing the transgene,
 - (ii) the embryo develops into the transgenic animal, and
 - (iii) the transgenic animal is viably born from the second female animal;

wherein the transgenic animal has the genetic sequence for the desired protein and is capable of being bred to produce offspring having cells stably containing the desired genetic sequence.

[0052] Chen *et al.* (1998) report the generation of several lines of transgenic mice that direct transgene expression to specific brain regions, including the striatum, cerebellum, CA1 region of the hippocampus, or deep layers of cerebral neocortex. These mice were

generated using a modified tetracycline-regulated system under the control of the neuron-specific enolase promoter. Transgene expression in these mice can be turned off completely with low doses of doxycycline (a tetracycline derivative) and driven to very high levels in the absence of doxycycline. The methods for obtaining the transgenic mice are described by Chen *et al.* (1998).

3. Methods of Identifying Agents that Modulate Bone Formation and Adipogenesis

[0053] The present invention provides a method for identifying agents that modulate bone formation. The method comprising administering a test agent and monitoring the expression of Δ FosB to determine whether the agent modulates bone formation, wherein an increase or decrease in the expression of Δ FosB expression indicates that the agent modulates bone formation. Moreover, an increase in the expression Δ FosB indicates that the agent is an inducer or promoter of bone formation, while a decrease in expression of Δ FosB indicates that the agent is an inhibitor of bone formation.

[0054] The present invention also provides a method for identifying agents that modulate adipogenesis. The method comprising administering a test agent and monitoring the expression of Δ FosB to determine whether the agent modulates adipogenesis or adipocyte differentiation, wherein an increase or decrease in the expression of Δ FosB expression indicates that the agent modulates adipogenesis. Moreover, an increase in expression Δ FosB indicates that the agent is an inhibitor of adipogenesis, while a decrease in expression of Δ FosB indicates that the agent is an inducer or promoter of adipogenesis.

[0055] As used herein, the term "agent" or "test agent" refers to any compound or molecule that is to be tested.

[0056] As used herein, the term "modulator" refers to any agent that alters the expression of a specific activity, such as bone formation, adipogenesis or Δ FosB expression. As used herein, the term "modulate" or "modulates" means alters or changes.

Thus, a test agent that modulates the expression of bone formation alters or changes bone formation, and a modulator of bone formation increases or decreases bone formation.

[0057] As used herein, the term “inducer” or “promoter” refers to any agent that induces, enhances, promotes or increases a specific activity, such as bone formation, adipogenesis, or Δ FosB expression.

[0058] As used herein the term “inhibitor” or “repressor” refers to any agent that inhibits, suppresses, represses, or decreases a specific activity, such as bone formation, adipogenesis, or Δ FosB expression.

[0059] Examples of agents of the present invention include but are not limited to peptides, small molecules, and antibodies. Agents can be randomly selected or rationally selected or designed. As used herein, an agent is said to be “randomly selected” when the agent is chosen randomly without considering the specific interaction between the agent and the target compound or site. As used herein, an agent is said to be “rationally selected or designed”, when the agent is chosen on a nonrandom basis which takes into account the specific interaction between the agent and the target compound or site and/or the conformation in connection with the agent’s action.

[0060] It is known that isoforms of Δ FosB are induced after many types of chronic perturbation such as exposure to cocaine, amphetamine, nicotine, opiates, antidepressants, and antipsychotics. Nestler *et al.* (1999) report that once induced Δ FosB isoforms persists in the brain for relatively long periods due to their extraordinary stability. Accordingly, agents that are rationally selected to modulate osteogenesis and adipogenesis include but are not limited to drugs of abuse, antidepressants, and antipsychotics. Specific examples of drugs of abuse include but are not limited to cocaine, amphetamine, nicotine, and opiate derivatives.

[0061] The methods of the present invention may be performed using *in vitro* cells (cultured cells) and cell lysates, specifically nuclear extracts. Examples of cells contemplated for identifying agents that modulate bone formation include but are not limited to calvarial cells, osteoblasts, osteoclasts, chondrocytes, and pluripotent precursor

cells, such as multipotent bone marrow stromal cells. Specific examples of osteoblast cell lines include MC3T3-E1, C2C12, MG-63 cells, U2OS cells, UMR106 cells, ROS 17/2.8 cells, SaOS2 cells, and the like are provided in the catalog from the ATCC. Additionally, bone and cartilage cell lines established from transgenic mice may be used. Examples of cells that are useful for identifying agents that modulate adipogenesis include but are not limited to adipocytes, preadipocytes, and pluripotent precursor cells, such as multipotent bone marrow stromal cells. Specific example of adipocyte cell lines include 3T3-L1 preadipocytes, 3T3 F422A, and ob 1771. Cell lysates and nuclear extracts may be obtained from the contemplated cells by methods routinely practiced (Schreiber *et al.*, 1989).

[0062] Further, US Patent 6,082,364 discloses pluripotent stem-like D1 cells, obtained from bone marrow, capable of differentiating into osteocytes, chondrocytes and adipocytes, depending on the environment encountered and treatment used. If systemically administered, the cells migrate to bone marrow. The cells may be transformed with recombinant DNA for the expression of both reporter genes and biological factors, such as growth factors. The patent teaches systemic administration of the cells to treat osteoporosis, osteolysis, and to improve bone implant adherence, augment bone growth or bone repair, augment cartilage repair, augment fat production for, *e.g.*, breast augmentation.

[0063] The methods of the present invention may also be performed using whole animals, preferably non-human transgenic animals. Preferred non-human transgenic animals include those that overexpress Δ FosB. Specific examples of such transgenic animals include animals carrying the TetOP- Δ FosB gene and bigenic NSE-tTA x TetOp- Δ FosB animals (Chen *et al.*, 1998). Most preferably, these animals are transgenic mice. In another embodiment, cells obtained from transgenic animals are used as the source of Δ FosB for identifying agents that modulate osteogenesis or adipogenesis.

[0064] Methods for determining whether a test agent alters the expression of Δ FosB include performing analyses and assays well known to the skilled artisan. Examples

include but are not limited to histochemical analyses, osteoclast like cell (OCL) formation assay, authentic osteoclast pit formation assay, Northern blot analysis, Western blot analysis, and electrophoretic mobility shift assays (EMSA) and supershift analysis. Other methods contemplated by the present invention for identifying test agents that modulate Δ FosB expression include PCR analyses, two hybrid screening assays (Fields *et al.*, (1989) *Nature* 340:245; Gyuris *et al.*, (1993) *Cell*, 75:791; Harper *et al.*, (1993) *Cell* 75:805; Serrano *et al.*, (1993) *Nature* 366:704; Hannon *et al.*, (1993) *Genes & Dev.* 7:2378) and reporter gene systems. In certain embodiments, the reporter gene can encode beta-galactosidase. In other embodiments, the reporter gene can encode chloramphenicol acetyltransferase (CAT), luciferase, and other reporter genes. For example, the reporter gene could be under the control of a promoter that Δ FosB interacts with. Thus, the expression of the reporter indicates the expression of Δ FosB.

[0065] Transgenic animals administered with the test agent can be sacrificed, and assays performed using specimens from the animals. Additionally, bone histomorphometric measurements (Parfitt *et al.*, 1987), serum osteocalcin measurements, urinary creatine measurements, serum leptin measurements, and marrow smears can be performed to determine whether the test agent alters the expression of Δ FosB.

[0066] It is within the skill of the artisan to perform side-by-side control experiments for determining whether a test agent modulates Δ FosB expression. An increase in Δ FosB expression as compared to the control indicates that the test agent is a potential promoter of bone formation, while a decrease in Δ FosB expression as compared to the control indicates that the test agent is a potential inhibitor of bone formation. Likewise, an increase in Δ FosB expression as compared to the control indicates that the test agent is a potential inhibitor of adipogenesis, while a decrease in Δ FosB expression as compared to the control indicates that the test agent is a potential inducer of adipogenesis.

[0067] The methods of the present invention may also be performed using a cell free system.

[0068] The methods of the present invention may be modified or performed in any available format, including high throughput assays. High throughput assays are useful for screening a large number of compounds in a given period of time. In one embodiment, assays can be formed using nucleic acids, wherein nucleic acids are placed on a DNA chip. In another embodiment, assays using cell-based screening are performed. US Patent 6,103,479 discloses miniature cell array methods and apparatus for cell-based screening. Methods have been described for making uniform micro-patterned arrays of cells for other applications, for example photochemical resist-photolithography (Mrksich and Whitesides, Ann. Rev. Biophys. Biomol. Struct. 25:55-78, 1996). US Patent 6,096,509 provides an apparatus and method for real-time measurement of a cellular response of a test compound on a flowing suspension of cells, in which a homogeneous suspension of each member of a series of cell types is combined with a concentration of a test compound, directed through a detection zone, and a cellular response of the living cells is measured in real time as the cells in the test mixture are flowing through the detection zone. The patent teaches the use of the apparatus in automated screening of libraries of compounds. The methods disclosed in the US Patents can be modified to determine whether test agents modulate the expression of Δ FosB using cells such as adipocytes or osteoblasts.

4. Methods of Using the Agents that Modulate the Expression of Δ FosB

[0069] The present invention is based in part on the finding that overexpression of Δ FosB in transgenic mice leads to increased bone formation throughout the skeleton and a continuous post-developmental increase in bone mass. The present invention is also based in part on the discovery that expression of Δ FosB inhibits adipogenesis both *in vitro* and *in vivo* and downregulates the expression of early markers of adipocyte differentiation. Additionally, the present invention is based in part on the conclusion that Δ FosB transcriptionally regulates osteoblastogenesis, since osteoblasts and adipocytes are thought to share a common precursor.

[0070] Osteogenesis is expected to be promoted by stimulating growth, differentiation, or activation of osteoblasts, while adipogenesis is expected to be promoted by stimulating growth, differentiation, or activation of adipocytes. Accordingly, an agent that modulates the expression of Δ FosB is potentially useful for modulating osteogenesis, adipogenesis, osteoblast proliferation and differentiation, adipocyte proliferation or differentiation, stromal stem cell differentiation, and pluripotent precursor cell differentiation. Thus, an agent that modulates the expression of Δ FosB is potentially useful for treating diseases or conditions associated with osteogenesis and adipogenesis and for regulating the differentiation and proliferation of osteoblasts and adipocytes.

[0071] As used herein, the term "osteogenesis" refers to bone formation.

[0072] As used herein, the term "adipogenesis" refers to the production of fat, the deposition of fat, or to the conversion of carbohydrate or protein to fat. The term is synonymous with "lipogenesis".

[0073] As used herein, the term "osteoblastogenesis" is the formation of osteoblasts.

[0074] As used herein, the term "differentiate" refers to having a different character or function from the original type of tissues or cells. Thus, "differentiation" is the process or act of differentiating.

[0075] As used herein, the term "proliferation" refers to the growth and production of similar cells.

[0076] As used herein, the term "treatment" refers to both therapeutic treatment and prophylactic, or preventative measures. The object of a treatment is to prevent or slow down (lessen) the disease or conditions and to ameliorate the symptoms.

[0077] In general, diseases and conditions associated with bone formation and metabolism include but are not limited to rickets, osteomalacia, osteopenia, osteosclerosis, renal osteodystrophy, osteoporosis (including senile and postmenopausal osteoporosis), Paget's disease, bone metastases, hypercalcaemia, hyperparathyroidism, osteopetrosis, periodontitis, and the abnormal changes in bone metabolism which may accompany rheumatoid arthritis and osteoarthritis. Some of these diseases are

characterized by insufficient bone formation or bone loss, while others involve an abnormal thickening or hardening of bone tissue. Examples of diseases that would benefit from inhibiting abnormal thickening of the bone include osteopetrosis and osteosclerosis. A specific example is Pycnodysostosis (PYCNO) a rare, autosomal recessive trait characterized by osteosclerosis, short stature, acro-osteolysis of distal phalanges, bone fragility, clavicular dysplasia and skull deformities with delayed suture closure (Maroteaux *et al.*, 1962, Presse Med. 70:999; Andren *et al.*, 1962, Acta. Chir. Scand. 124:496). Therefore, agents identified as inhibitors of bone formation are potentially useful for treating PCYNO and other diseases or conditions characterized by osteosclerosis and osteopetrosis.

[0078] There are also many diseases and conditions characterized by the need to enhance bone formation. The most obvious is the case of bone fractures, where it would be desirable to stimulate bone growth and to hasten and complete bone repair. Agents that enhance bone formation are potentially useful in facial reconstruction procedures. Other bone deficit conditions include bone segmental defects, periodontal disease, metastatic bone disease, osteolytic bone disease and conditions where connective tissue repair would be beneficial, such as healing or regeneration of cartilage defects or injury. Also of great significance is the chronic condition of osteoporosis, including age-related osteoporosis and osteoporosis associated with post-menopausal hormone status. Other conditions characterized by the need for bone growth include primary and secondary hyperparathyroidism, disuse osteoporosis, diabetes-related osteoporosis, and glucocorticoid-related osteoporosis.

[0079] In one embodiment of the invention, agents identified as inducers or stimulators of bone formation are potentially useful for repairing bone defects and deficiencies, for promoting bone healing in plastic surgery, stimulating bone ingrowth into non-cemented prosthetic joints and dental implants, elevating peak bone mass in pre-menopausal women, treating growth deficiencies, treating periodontal disease and defects, and other tooth repair processes, treating skeletal disorders, such as age-related osteoporosis,

post-menopausal osteoporosis, glucocorticoid-induced osteoporosis or disuse osteoporosis and arthritis, or any condition that benefits from stimulation of bone formation. In an alternative embodiment of the invention, these agents by themselves or in combination with other agents are potentially useful in the repair of congenital, trauma-induced or surgical resection of bone (for instance, for cancer treatment), and in cosmetic surgery. In another embodiment of the invention, the agents identified by the method of the present invention and combinations thereof can be used for limiting or treating cartilage defects or disorders, and may be useful in wound healing or tissue repair.

[0080] Moreover, the agents identified as modulators of osteogenesis are potentially useful for enhancing or preventing the proliferation of osteoblasts, osteoclasts, and chondrocytes *in vitro*, depending on whether the agent is an inducer or an inhibitor of bone formation. These agents are also useful for inducing or inhibiting differentiation of pluripotent precursor cells, preferably bone marrow stromal stem cells, into osteoblasts. It is within the skill of the artisan to administer an agent to isolated cells in culture and to observe differentiation or proliferation of the *in vitro* cells.

[0081] Generally, diseases associated with adipogenesis include body weight disorders such as obesity and cachexia, and nonshivering and shivering thermogenesis. Accordingly, in one aspect of the invention, the agents identified as modulators of adipogenesis are potentially useful for modulating body weight-related processes, including, for example, treatment of body weight disorders such as obesity and cachexia, and thermogenesis. Depending on the desired result, an agent identified to induce adipogenesis is potentially useful for increasing body weight and an agent identified to prevent adipogenesis is potentially useful for decreasing body weight.

[0082] Adipogenesis when it occurs in the bone marrow is deleterious because the bone becomes necrotic. Such bones are weak and prone to fracturing. However, adipogenesis in other locations of the body may be highly desirable, for example, in the breast for breast augmentation. Pluripotent bone marrow cells may be implanted directly into breast

fat pouches. Agents that are identified as capable of inducing the differentiation of pluripotent bone marrow cells into adipocytes may be administered to increase the production of fat cells in the breast.

[0083] The agents identified as modulators of adipogenesis may also be used to induce or inhibit proliferation or differentiation of isolated preadipocytes or adipocytes in culture, for example 3T3-L1, 3T3 F422A, ob 1771, or preadipocytes and adipocytes from transgenic animals that can be induced to overexpress Δ FosB. It is within the skill of the artisan to administer the test agent to the isolated preadipocytes or adipocytes and to observe the proliferation or differentiation of the *in vitro* cells.

5. Pharmaceutical Compositions and Methods of Delivery

[0084] The test agents and agents or compounds identified to be modulators of Δ FosB expression can be administered to subjects either by themselves or in pharmaceutical compositions. As used herein, the term "pharmaceutical composition" refers to a composition comprising an agent together with a pharmaceutically acceptable carrier or diluent. As used herein, the term "carrier" in a composition refers to a diluent, adjuvant, excipient, or vehicle with which the agent or compound is mixed.

[0085] Pharmaceutical carriers are chosen such that side effects from the pharmaceutical compound are minimized and the performance of the agent is not canceled or inhibited to such an extent that the treatment or the function of the agent is ineffective. Carriers can also be used to facilitate administration of the compound, for example, to increase the solubility of the compound. A pharmaceutically acceptable carrier includes, but is not limited to, physiological saline, ringers, phosphate buffered saline, calcium carbonate, calcium phosphate, various sugars or types of starch, cellulose derivatives, gelatin, vegetable oils, and polyethylene glycols. Pharmaceutical compositions comprising test agents or agents identified by the present invention, can be in either solid or liquid form.

[0086] Solid form preparations of pharmaceutical compositions include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. The term "preparation" is intended to include the formulation of the agent with encapsulating material as a carrier, providing a capsule in which the active component with or without other carriers, is surrounded by a carrier. Similarly, cachets and lozenges are included. Tablets, powders, capsules, pills, cachets, and lozenges can be used as solid dosage forms suitable for oral administration. A solid carrier can be one or more substances which may also act as diluents, flavoring agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material. In powders, the carrier is a finely divided solid which is in a mixture with the finely divided active agent. In tablets, the active agent is mixed with the carrier having the necessary binding properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain from five or ten to about seventy percent of the active agent or compound. Suitable carriers include magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, a low melting wax, cocoa butter, and the like.

[0087] For preparing suppositories, a low melting wax, such as a mixture of fatty acid glycerides or cocoa butter, is first melted and the active component is dispersed homogeneously therein, as by stirring. The molten homogeneous mixture is then poured into convenient sized molds, allowed to cool, and thereby to solidify.

[0088] Liquid form preparations include solutions, suspensions, and emulsions, for example, water or water propylene glycol solutions. For parenteral injection liquid preparations can be formulated in solution in aqueous polyethylene glycol solution.

[0089] Aqueous solutions suitable for oral use can be prepared by dissolving the active component in water and adding suitable colorants, flavors, stabilizing and thickening agents as desired. Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, such as natural or

synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, and other well-known suspending agents.

[0090] Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations for oral administration. Such liquid forms include solutions, suspensions, and emulsions. These preparations may contain, in addition to the active agent, colorants, flavors, stabilizers, buffers, artificial and natural sweeteners, dispersants, thickeners, solubilizing agents, and the like.

[0091] The test agents and agents or compounds identified to modulate Δ FosB expression can be administered to the subject as pharmaceutically acceptable salts. Examples of pharmaceutically acceptable salts include acid addition salts such as those containing hydrochloride, sulfate, phosphate, sulfamate, acetate, citrate, lactate, tartrate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, cyclohexylsulfamate and quinate. (See *e.g.*, PCT/US92/03736). Such salts can be derived using acids such as hydrochloric acid, sulfuric acid, phosphoric acid, sulfamic acid, acetic acid, citric acid, lactic acid, tartaric acid, malonic acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid, cyclohexylsulfamic acid, and quinic acid.

[0092] Pharmaceutically acceptable salts can be prepared by standard techniques. For example, the free base form of the compound is first dissolved in a suitable solvent such as an aqueous or aqueous-alcohol solution, containing the appropriate acid. The salt is then isolated by evaporating the solution. In another example, the salt is prepared by reacting the free base and acid in an organic solvent.

[0093] The present invention provides methods of administering a test agent in the form of a pharmaceutical composition to subjects to determine whether the agent is effective in modulating Δ FosB expression, osteogenesis, or adipogenesis. Moreover, the present invention provides methods of administering a pharmaceutical composition comprising an agent identified to modulate Δ FosB expression to treat subjects diagnosed with diseases or conditions associated with osteogenesis or adipogenesis. In treating a patient

exhibiting a disorder associated with osteogenesis or adipogenesis, a therapeutically effective amount of an agent or agents is administered. A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient. For example, an effective amount for therapeutic uses is the amount of the composition comprising an active agent that provides a clinically significant increase in healing rates in fracture repair; reversal of bone loss in osteoporosis; reversal of cartilage defects or disorders; prevention or delay of onset of osteoporosis; stimulation and/or inhibition of bone formation in fracture non-unions and distraction osteogenesis; increase and/or decrease in bone growth into prosthetic devices; repair of dental defects; increase or decrease in adipogenesis or obesity; and increase or decrease in loss of appetite. Such effective amounts will be determined using routine optimization techniques and are dependent on the particular condition to be treated, the condition of the patient, the route of administration, the formulation, and the judgment of the practitioner and other factors evident to those skilled in the art. The dosage required for the compounds of the invention (for example, in osteoporosis where an increase in bone formation is desired) is manifested as a statistically significant difference in bone mass between treatment and control groups. This difference in bone mass may be seen, for example, as a 5-20% or more increase in bone mass in the treatment group. Other measurements of clinically significant increases in healing may include, for example, tests for breaking strength and tension, breaking strength and torsion, 4-point bending, increased connectivity in bone biopsies and other biomechanical tests well known to those skilled in the art. General guidance for treatment regimens is obtained from experiments carried out in animal models of the disease of interest.

[0094] Depending on the specific conditions being treated, agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, Pa. (1990). Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular,

subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few. Some methods of delivery that may be used include but are not limited to encapsulation in liposomes, transduction by retroviral vectors, localization to nuclear compartment utilizing nuclear targeting site found on most nuclear proteins, transfection of cells *ex vivo* with subsequent reimplantation or administration of the transfected cells, and a DNA transporter system.

[0095] Toxicity and therapeutic efficacy of agents or compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀ /ED₅₀. Agents or compounds which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such agents or compounds lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

[0096] For any agent used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ as determined in cell culture (*i.e.*, the concentration of the test compound which achieves a half-maximal disruption of the protein complex, or a half-maximal inhibition of the cellular level and/or activity of a complex component). Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by HPLC. The exact formulation, route of administration, and dosage can be chosen by the individual physician in view of the patient's condition. (See *e.g.* Fingl *et al.*, in *The Pharmacological Basis of Therapeutics*,

1975, Ch. 1 p. 1). It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

[0097] In general, the agents utilized in the pharmaceutical method of this invention can be administered to a typical human on a daily basis as an oral dose of about 0.1 mg/kg-1000 mg/kg, and more preferably from about 1 mg/kg to about 200 mg/kg. The parenteral dose will appropriately be about 20-100% of the oral dose. While oral administration may be preferable in most instances (for reasons of ease, patient acceptability, and the like), alternative methods of administration may be appropriate for selected compounds and selected defects or diseases. The dosages, however, may be varied depending upon the requirements of the patient, the severity of the condition being treated, and the agent being employed. As discussed above, determination of the proper dosage for a particular situation is within the skill of the art. Generally, treatment is initiated with smaller dosages which are less than the optimum dose of the compound. Thereafter, the dosage is increased by small increments until the optimum effect under the circumstances is reached. For convenience, the total daily dosage may be divided and administered in portions during the day, if desired.

[0098] Further, the agents or compounds for treating diseases and conditions identified by the present invention may also be co-administered with other well known therapeutic agents that are selected for their particular usefulness against the condition that is being treated. The agents can be combined with estrogens or estrogen-related compounds since

estrogens are known to inhibit bone resorption. Typical estrogen compounds include estradiol, progesterone, and analogs thereof as are well known in the art. Other compounds include but are not limited to bisphosphonates and related compounds such as those set forth in U.S. Pat. No. 5,312,814, calcium supplements (Prince, R. L. *et al.*, 1991, N. Engl. J. Med. 325:1189), vitamin D supplements (Chapuy, M. C. *et al.*, 1992, N. Engl. J. Med. 327:1637), sodium fluoride (Riggs, B. L. *et al.*, 1992, N. Engl. J. Med. 327:620), androgen (Nagent de Deuxchaisnes, C., 1983, in Osteoporosis, a Multi-Disciplinary Problem, Royal Society of Medicine International Congress and Symposium Series No. 55, Academic Press, London, p. 291), and calcitonin (Christiansen, C., 1992, Bone 13 (Suppl. 1):S35).

[0099] Additionally, the agents or compounds identified in the present invention may also be co-administered with compounds effective for modulating obesity, anorexia, cachexia, and other diseases or conditions associated with excessive increase in weight or loss of appetite. US Patent 6,068,976, discloses a down regulator of ob gene expression, BRL49653, which increases food intake and body weight in rats. The administration of an effective amount of an ob gene down regulator will be able to treat a patient suffering from anorexia, cachexia and other wasting diseases characterized by loss of appetite, diminished food intake or body weight loss. Additionally, it is known that glucocorticoids, have the properties of decreasing food consumption and body weight gain in rats. The administration of an effective amount of an ob gene up regulator will be able to treat a patient suffering from excessive food consumption and obesity, and related pathological conditions such as type II adult onset diabetes, infertility (Chehab, *et al.*, Nature Genetics, 12:318-320, 1996), hypercholesterolemia, hyperlipidemia, cardiovascular diseases and hypertension. Thus, the present invention contemplates administering pharmaceutical compositions comprising agents identified as modulators of Δ FosB expression in combination with agents known to regulate weight and appetite for the treatment of diseases or conditions associated with excessive weight loss or gain and loss or increase in appetite.

[00100] The administration of these additional compounds may be simultaneous with the administration of the agents identified to be modulators of Δ FosB expression or may be administered in tandem with the administration of the compounds of the invention. Any suitable protocol may be devised whereby the various compounds to be included in the combination treatment are administered within minutes, hours, days, or weeks of each other. Repeated administration in a cyclic protocol is also envisioned.

6. Method of Identifying Genes Associated with Δ FosB Expression

[0100] The present invention also contemplates methods of identifying genes that are associated with Δ FosB expression, *i.e.*, genes that may be regulated by Δ FosB expression or regulate Δ FosB expression. The present invention also contemplates identifying genes that are associated with osteogenesis and adipogenesis. Additionally, the present invention provides methods for the identification of compounds that modulate the expression of genes or the activity of gene products associated with abnormal bone formation or abnormal adipogenesis.

[0101] In higher organism, the expression of certain genes in a cell determines the life processes, *e.g.* development and differentiation, homeostasis, response to insults, cell cycle regulation, aging, apoptosis, *etc.*, to be carried out by the cell. Alterations in gene expression changes the course of development of a normal cell. Clearly, which genes are expressed has a profound effect on the nature of any given cell. Accordingly, methods for analyzing gene expression are critical to basic molecular biological research.

Identification of differentially-expressed genes can provide a key to diagnosis, prognosis, and treatment of a variety of diseases or condition states in animals, including humans, and plants. Additionally, these methods can be used to identify differentially-expressed sequences due to changes in gene expression level associated with predisposition to disease or conditions, preferably associated with osteogenesis or adipogenesis.

Identification of such genes helps in development of new drugs and diagnostic methods for treating or preventing the occurrence of such diseases.

[0103] As used herein, the term “differential expression” refers to both quantitative as well as qualitative differences in the genes’ temporal and/or tissue expression patterns. Thus, a differentially expressed gene may qualitatively have its expression activated or completely inactivated in normal versus abnormal bone formation state or abnormal body weight state, or under control versus experimental conditions. Such a qualitatively regulated gene will exhibit an expression pattern within a given tissue or cell type which is detectable in either control subject or subjects with a disorder, but is not detectable in both. Alternatively, such a qualitatively regulated gene will exhibit an expression pattern within a given tissue or cell type which is detectable in either control or experimental subjects, but is not detectable in both. As used herein, “detectable” refers to an RNA expression pattern which is detectable via the standard techniques of differential display, RT-PCR and/or Northern analyses, which are well known to those of skill in the art.

32

[0105] In order to identify differentially expressed genes, RNA, either total or mRNA, may be isolated from the tissues of the animals or from the cells described above. RNA samples are obtained from tissues of experimental subjects and from corresponding tissues of control subjects. Any RNA isolation technique which does not select against the isolation of mRNA may be utilized for the purification of such RNA samples. See, for example, Ausubel, F. M. *et al.*, eds., 1987-1993, Current Protocols in Molecular Biology, John Wiley & Sons, Inc. New York, which is incorporated herein by reference in its entirety. Additionally, large numbers of tissue samples may readily be processed using techniques well known to those of skill in the art, such as, for example, the single-step RNA isolation process of Chomczynski, P. (1989, U.S. Pat. No. 4,843,155), which is incorporated herein by reference in its entirety.

[0106] Transcripts within the collected RNA samples which represent RNA produced by differentially expressed genes may be identified by utilizing a variety of methods which are well known to those of skill in the art. For example, differential screening (Tedder *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:208-212), subtractive hybridization (Hedrick *et al.*, 1984, Nature 308:149-153; Lee *et al.*, 1984, Proc. Natl. Acad. Sci. USA 88:2825), and, preferably, differential display (Liang, P. and Pardee, A. B., 1992, Science 257:967-971; U.S. Pat. No. 5,262,311, which is incorporated herein by reference in its entirety), may be utilized to identify nucleic acid sequences derived from genes that are differentially expressed.

[0107] US Patent 5,968,784 provides a method for tagging and identifying all of the expressed genes in a given cell population. This method thus allows even mRNAs with low copy number to be detected. By comparing gene expression profiles among cells, this method may be used to identify individual genes whose expression is associated with a pathological phenotype. Using high throughput DNA sequencing and associated information system support to analyze such DNA sequencing, the disclosed method also permits the generation of global gene expression profiles in a reasonable length and time. Thus, the patent provides a simple and rapid method of obtaining sufficient data to use in

an information system known to those of skill in the art to obtain global gene expression profile and identify genes of interest.

[0108] Once potentially differentially expressed gene sequences have been identified via bulk techniques such as, for example, those described above, the differential expression of such putatively differentially expressed genes should be corroborated. Corroboration may be accomplished via, for example, such well known techniques as Northern analysis, quantitative RT PCR or RNase protection. Upon corroboration, the differentially expressed genes may be further characterized and identified.

7. Methods of Inhibiting Δ FosB Expression Using Nucleic Acids

[0109] The present invention also provides antisense nucleic acids and ribozymes which exhibit the ability to modulate osteogenesis and adipogenesis. Such molecules are designed to reduce or inhibit Δ FosB gene activity. Techniques for the production and use of such molecules are well known to those of skill in the art.

[0110] Antisense RNA and DNA molecules act to directly block the translation of mRNA by hybridizing to targeted mRNA and preventing protein translation. Antisense approaches involve the design of oligonucleotides that are complementary to a target gene mRNA. The antisense oligonucleotides will bind to the complementary target gene mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required.

[0111] As used herein, a sequence "complementary" to a portion of an RNA, refers to a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as

the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

[0112] Oligonucleotides that are complementary to the 5' end of the message, *e.g.*, the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have also been shown to be effective at inhibiting translation of mRNAs as well (Wagner, R., 1994, Nature 372:333-335).

[0113] Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5', 3' or coding region of target gene mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

[0114] The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, *etc.* The antisense oligonucleotide may comprise a modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil,

2-methylthio-N6-isopentenyladenine, wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, and 2,6-diaminopurine.

[0115] The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose. In yet another embodiment, the antisense oligonucleotide may comprise at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

[0116] Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.* by use of an automated DNA synthesizer (such as those that are commercially available from Biosearch, Applied Biosystems, *etc.*). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein *et al.* (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.*, 1988, Proc. Natl. Acad. Sci.U.S.A. 85:7448-7451), *etc.*

[0117] A number of methods have been developed for delivering antisense DNA or RNA to cells, *e.g.*, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA

constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

[0118] However, it is often difficult to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs. Therefore a preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous target gene transcripts and thereby prevent translation of the target gene mRNA. For example, a vector can be introduced *in vivo* such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto *et al.*, 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster *et al.*, 1982, Nature 296:39-42), *etc.* Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct which can be introduced directly into the tissue site. Alternatively, viral vectors can be used which selectively infect the desired tissue, in which case administration may be accomplished by another route (*e.g.*, systemically).

[0119] Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, J. Amer. Med. Assn., 260:3030, 1988). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

[0120] There are two basic types of ribozymes namely, tetrahymena-type (Hasselhoff, Nature, 334:585, 1988) and "hammerhead"-type. Tetrahymena-type ribozymes recognize sequences which are four bases in length, while "hammerhead"-type ribozymes recognize base sequences 11-18 bases in length. The longer the recognition sequence, the greater the likelihood that sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to tetrahymena-type ribozymes for inactivating a specific mRNA species and 18-based recognition sequences are preferable to shorter recognition sequences

8. Genetic Therapy

[0121] As discussed above, the present invention provides methods of identifying genes associated with osteogenesis, adipogenesis, or Δ FosB expression, including genes that are regulated by Δ fosB expression and that regulates Δ fosB expression. The present invention also contemplates a method of delivering nucleic acid molecules, such as the Δ FosB nucleic acids, to target sites for the treatment of diseases associated with osteogenesis and adipogenesis.

[0122] Gene therapy is a method for delivering functionally active therapeutic or other forms of genes into targeted cells. Initial efforts of gene transfer into somatic tissues have relied on indirect means called *ex vivo* gene therapy, wherein target cells are removed from the body, transfected or infected with vectors carrying recombinant genes, and re-implanted into the body. Techniques currently used to transfer DNA *in vitro* into cells

include calcium phosphate-DNA precipitation, DEAE-Dextran transfection, electroporation, liposome mediated DNA transfer or transduction with recombinant viral vectors. These transfection protocols have been used to transfer DNA into different cell types including epithelial cells (U.S. Pat. No. 4,868,116), endothelial cells (WO89/05345), hepatocytes (Ledley *et al.*, 1987 Proc Natl Acad Sci U S A. 84(15):5335-9) fibroblasts (U.S. Pat. No. 4,963,489), lymphocytes (U.S. Pat. No. 5,399,346;) and hematopoietic stem cells (U.S. Pat. No. 5,399,346).

[0123] Direct *in vivo* gene transfer has been carried out with formulations of DNA trapped in liposomes (Seol *et al.*, 2000 In Vivo 14(4):513-7, or in proteoliposomes that contain viral envelope receptor proteins (Gould-Fogerite *et al.*, 1989, Gene 84(2):429-38), and with DNA coupled to a polylysine-glycoprotein carrier complex. In addition, "gene guns" have been used for gene delivery into cells (Australian Patent No. 9068389). Lastly, naked DNA, or DNA associated with liposomes, can be formulated in liquid carrier solutions for injection into interstitial spaces for transfer of DNA into cells (WO90/11092).

[0124] Viral vectors are often the most efficient gene therapy system, and recombinant replication-defective viral vectors have been used to transduce (*i.e.*, infect) cells both *ex vivo* and *in vivo*. Such vectors include retroviral, adenovirus and adeno-associated and herpes viral vectors. Accordingly, in one embodiment the nucleic acids including genes, ribozymes, and antisense molecules associated with adipogenesis, osteogenesis, or Δ FosB expression can be subcloned into an appropriate vector and transferred into a cell or tissue by gene transfer techniques discussed above.

[0125] In another embodiment, the nucleic acids associated with adipogenesis, osteogenesis, or Δ FosB expression can be provided to the cell or tissue using a transfection facilitating composition, such as cationic liposomes containing desired polynucleotides.

[0126] In light of the foregoing general discussion, the specific examples presented below are illustrative only and are not intended to limit the scope of the invention. Other generic and specific configurations will be apparent to those persons skilled in the art.

EXAMPLES

Materials and Methods

[0127] *Histomorphometric and Biochemical Analyses:* Ten week old 1A, 11A, and control mice were injected with calcein (20mg/kg, Sigma MO) at 10 and 3 days before sacrifice, respectively, to label bone mineralization fronts. Mice were bred from heterozygous breeders, providing bitransgenic Δ FosB-expressing mice and monotransgenic non-expressing littermate controls. Specimens were fixed in 3.7% formaldehyde/PBS and embedded by standard procedures in methylmethacrylate resin. Five micromolar toluidine blue or 10 μ m unstained sections were used for standard bone histomorphometric measurements (Parfitt *et al.*, 1987) using the Osteomeasure system (OsteoMetrics, GA). Serum osteocalcin was measured by radioimmunoassay (Biomedical Technologies Inc, MA). Urinary deoxypyridinoline crosslinks were measured by the Pylinks-D assay (Metra Biosystems Inc, CA); urinary creatinine was measured enzymatically on a clinical chemistry analyser (BM/Hitachi 717, CA). Serum leptin was measured by a mouse specific immunoassay (Quantikine M, R&D Systems, Minneapolis). Marrow smears were carried out on humeri from control and 1A mice. The joints were removed and marrow was flushed out onto microscope slides using minimal essential medium Eagle, α -modification (α -MEM, Sigma), followed by Oil Red O and 1% toluidine blue staining. Adipocytes and total marrow cells were counted on five randomly selected fields of each smear from 4 mice of each genotype. Adipocyte number is expressed as a percentage of marrow cell number to allow for the reduction in bone marrow in the 1A mice. Data were analyzed by one-way ANOVA and Fisher's post-hoc test, with $p < 0.05$ considered statistically significant.

[0128] Cell Culture and DNA Transfections: Primary murine calvarial cell cultures were prepared from 1-2 day old control or NSE- Δ FosB 11A mice and maintained as described by Bellows *et al.* (1990). Homozygous bitransgenic 11A litters were bred. The same breeder male and a non-transgenic female were used to provide monotransgenic non-expressing controls. At confluency, cultures were supplemented with 50 μ g/ml ascorbic acid (Wako, Japan), 5mM β -glycerophosphate (Sigma) and 10nM dexamethasone (Sigma). Bone marrow cells were flushed from femora and tibiae of 4 week old mice and maintained in DMEM (Sigma) supplemented with 10% FBS and 1% penicillin/streptomycin. After 5 days in culture, plates were washed free of non-adherent cells and the adherent layer was cultured until confluent. Cells were detached and replated at a density of 2×10^4 /cm². At confluency, cells were incubated with either osteoblastic or adipocytic-inducing agents as described (Quarles *et al.*, 1992) before harvesting for experiments. The C2C12 myoblasts were maintained as described previously (Cao *et al.*). The osteoblastic phenotype was induced by incubating in DMEM, supplemented with 5% FBS and 300ng/ml human recombinant BMP-2 for 48hours. The MC3T3-E1 (pre-osteoblastic) and 3T3-L1 (pre-adipocytic) cell lines were purchased from ATCC and maintained according to standard protocols; the osteoblastic or adipocytic phenotype respectively was induced as described in Quarles *et al.* (1992) and Cao *et al.* (1991). Cells were transfected using lipofectamine (GIBCO BRL) according to the manufacturer's instructions. In transient expression studies, cells were harvested 48-72 hours post-transfection. To establish stable transfectants, cells were split 1:10, 72 hours post-transfection and selected in 0.5mg/ml G418 (GIBCO) for 3-4 weeks.

[0129] Osteoclast Like Cell (OCL) Formation Assay: OCLs were generated as previously described by Aoki *et al.* (1993), then fixed and stained for tartrate-resistant acid phosphatase (TRAP; 34) and TRAP-positive multinucleated OCLs (nuclei > 3) were counted.

[0130] *Authentic Osteoclast Pit Formation Assay:* In vitro bone resorption was assayed as described (Aoki *et al.*, 1993). Resorption pits were measured using an Osteomeasure analysis system (Osteometrics, GA). Total pit area and number for each dentin slice were measured.

[0131] *RNA Extraction and Northern Blot Analysis:* Total RNA was extracted from cells with TRIZOL (GIBCO) according to manufacturer instructions. 20µg total RNA were resolved in 1% denaturing agarose/formaldehyde gels and transferred onto Hybond-N nylon membranes (Amersham) as described (Sambrook *et al.*, 1989). Membranes were sequentially hybridized with ³²P-labelled cDNA probes for mouse osteocalcin, osteopontin, and collagen type 1. The C/EBP probe was obtained by RT-PCR using conditions described previously (Sabakatos *et al.*, 1998).

[0132] *Protein Extracts and Western Blot Analysis:* Nuclear extracts from cells were prepared as described previously (Schreiber *et al.*, 1989). Total cellular protein from tissues was prepared by sonication in modified RIPA buffer supplemented with proteinase and phosphatase inhibitors (REF). Protein concentration was determined by BCA assay (PIERCE, IL). Protein samples were analyzed by Western blot followed by chemiluminescent detection (Amersham, NJ) using standard techniques. All antibodies used were purchased from Santa Cruz Biotechnology (CA), except for OSF2/CBFA1.

[0133] *Histochemical Analysis of Osteoblast Cultures:* Calvarial osteoblast cultures were stained with alkaline phosphatase (Sigma) for osteoblasts and Oil Red O (Sigma) for adipocyte lipid. For measurement of adipocytes and mineralization, cultures were set up in 24 well plates and stained with Oil Red O and by a modified Von Kossa technique (Baron *et al.*, 1983). The same wells were used to measure both percent mineralization and adipocyte number. Adipocytes were counted from 5 random fields of view under a light microscope at the 40x objective (0.1963mm² view area). Mineralizing area was

measured across the entire well. Three wells were measured at each time point in each of 2 experiments.

546 a' → [0134] ~~*Electrophoretic Mobility Shift Assays (EMSA) and Supershift Analysis:*~~ EMSA was performed using a ³²P-labelled double stranded oligonucleotide corresponding to the human collagenase TPA-response element, which contains an AP-1-binding site (GTCGACGTGAGTCAGCGCGC), as described (Sabakatos *et al.*, 1998). For supershift analysis, antibodies were incubated with nuclear extracts for 30 min on ice prior to addition of ³²P-labelled oligonucleotide. Following electrophoresis, gels were dried and the complexes were visualized by autoradiography.

Example 1. NSE- FosB is Expressed in Transgenic Mice Exhibit Markedly Denser Bones

[0135] The present invention is based in part on the finding that NSE-FosB expressed in transgenic mice exhibits denser bones. Although NSE-tTA (line A) x TetOp- ΔFosB (line 11) bitransgenic animals were initially generated to study the role of ΔFosB in the brain (Chen *et al.*, 1998), showing inducible expression of ΔFosB in specific brain regions as well as altered behavioral responses (Kelz *et al.*, 1999), it was observed that NSE (data not shown), and consequently ΔFosB (Fig 1B), were expressed in osteoblasts, chondrocytes and adipocytes, when the animals were kept off doxycycline, but not in osteoclasts (data not shown). Transgenic mice kept off doxycycline from conception, leading to ΔFosB expression throughout development, were found to have strikingly increased bone density. This phenotype was even more dramatic in mice derived from another TetOp- ΔFosB founder line (line 1), which is known to express much higher levels of FosB (Chen *et al.*, 1998). X-ray analysis of these NSE-ΔFosB founder lines showed that bone density was markedly increased throughout the skeleton in mature mice (Fig. 1A). As anticipated from the patterns of NSE expression, both lines expressed high levels of FosB isoforms (Fig. 1B) not only in brain, but also in bone (calvariae) and adipose tissue, and to a lesser extent in spleen and skin (Fig. 1C). Although significantly

elevated in osteoblast preparations from the transgenic mice, Δ FosB isoforms were also expressed at detectable levels in wild-type osteoblasts (Fig. 1C).

[0136] NSE- Δ FosB mice were indistinguishable from control littermates at birth, but with advancing age trabecular bone volume increased markedly until the marrow space was almost entirely filled with lamellar bone (15 week-old 1A mice), (Fig. 2A) leading to extramedullary hematopoiesis and splenomegaly. The increased bone mass was proportional to gene dosage, since trabecular bone volume, although significantly increased in 11A mice, was much higher in 1A mice (Fig. 2B). Furthermore, the increased bone mass could be induced in mature animals by removing doxycycline or reversed by two weeks of doxycycline treatment (data not shown). These data exclude the possibility that the observed skeletal phenotype could be the consequence of developmental alterations. Calvarial bone thickness was also increased, indicating that bone derived from either intramembranous or endochondral ossification were equally affected. In contrast to c-Fos overexpressing mice (Grigoriadis *et al.*, 1993, Wang *et al.*, 1995), osteosarcoma was never observed. Osteoblasts were orderly, fluorochrome labels were sharp, and there was no marrow fibrosis or woven bone, indicating that bone formation, although markedly increased, is still a controlled process in these transgenic animals.

Example 2. Increased Bone Density *in vivo* is Due to Increased Bone Formation

[0137] The present invention is also based in part on the finding that increased bone density *in vivo* is due to increased bone formation. Possible pathways by which bone mass can increase *in vivo* all result from an imbalance between bone resorption and formation in favor of bone formation. Firstly, bone mass may be elevated due to impaired osteoclast function, resulting in an osteopetrosis characterized by the presence of many thin unremodelled trabeculae containing growth plate cartilage, and impaired tooth eruption (Soriano *et al.*, 1991, Hayman *et al.*, 1996, Seifert *et al.*, 1985). A morphologically similar phenotype is observed when bone resorption is reduced due to

impaired osteoclast differentiation (Johnson *et al.*, 1992, Wang *et al.*, 1992, Franzoso *et al.*, 1997, Tondravi *et al.*, 1997, Marks *et al.*, 1976, Simonet *et al.*, 1997). The third mechanism by which bone mass can increase *in vivo* is via increased bone formation (osteosclerosis). A phenotype as dramatic as the one reported here has however never been described. Only mild increases in bone density have been documented, following targeted disruption of the osteocalcin gene (Ducy *et al.*, 1996), after prolonged treatment with PTH or PGE₂17,18, or more recently in the leptin signaling-deficient ob/ob and db/db mice (Ducy *et al.*, 2000).

[0138] Detailed static and dynamic histomorphometric analysis of proximal tibiae and biochemical analyses of 10-week old 11A mice demonstrated that the increased bone mass in NSE- Δ FosB mice results from increased bone formation rather than impaired bone resorption. Histomorphometric markers of osteoblast proliferation and function were increased, including serum osteocalcin levels, which reflects bone formation at the systemic level (Fig. 2B). In addition, the expression of genes encoding bone matrix proteins was also up-regulated in calvariae from NSE- Δ FosB mice (Fig. 2B). In contrast, neither osteoclast surface or number, nor urinary deoxypyridinoline crosslinks were increased, demonstrating that bone resorption was not altered *in vivo* (Fig. 2C). *In vitro* bone resorption assays carried out on isolated NSE- FosB (11A) osteoclasts revealed no difference in pit number or size (Fig. 2C). Similarly, reciprocal co-culture experiments using osteoblasts and bone marrow from NSE- FosB (11A) or control animals, demonstrated no significant difference in osteoclastogenic potential of FosB marrow or osteoblasts (Fig. 2C). Tartrate-resistant acid phosphatase (TRAP) staining appeared normal in both neonate and mature mice. Finally, tooth eruption was neither impaired nor delayed, trabecular and cortical bone appeared normal at birth, and cartilage remnants were not detected within the secondary spongiosa, thereby further excluding osteopetrosis. Thus, the main cellular defect in these mice is a marked increase in bone formation, leading progressively to severe osteosclerosis.

Example 3. The Increase in Bone Formation is Cell-Autonomous in the Δ FosB Transgenic Osteoblast Lineage

[0139] The present invention is also based on the finding that bone formation is cell autonomous in Δ FosB transgenic osteoblast lineage. To determine whether the increased bone formation in NSE- Δ FosB mice was inherent to the osteoblast lineage or the result of systemic or micro-environmental changes, primary calvarial cultures were generated and differentiated *in vitro*. NSE- Δ FosB cultures demonstrated earlier and more rapid mineralized nodule formation, as well as increased expression of marker genes associated with the osteoblast phenotype, thus establishing a cell-autonomous effect of Δ FosB on osteoblasts and bone formation (Figs. 3A-D).

[0140] Osteoblasts and adipocytes are believed to originate from a common mesenchymal precursor and are both found in long-term murine primary calvarial cultures (Bellows *et al.*, 1994). A significant reduction in adipocyte number in calvarial cultures was observed from NSE- Δ FosB mice. The few adipocytes that differentiated in the NSE- Δ FosB cultures were less mature, with only few and small lipid droplets, in contrast to the large, numerous droplets observed in control calvarial cultures (Figs. 4A,B). Consistently, expression of transcription factors associated with the adipocytic phenotype such as PPAR2, C/EBP and C/EBP was also decreased relative to wild-type cultures at all time points studied (Fig. 4C).

[0141] Confirming changes observed *in vitro*, abdominal fat levels were decreased in NSE- Δ FosB mice (Fig. 4D) and marrow smears demonstrated reduced adipocyte numbers (mean adipocytes/marrow cells (%) \pm SEM: control, 14.2 ± 2 ; 11A, 2.3 ± 1 , $p < 0.05$). Moreover, *in vitro* cultures of bone marrow stromal cells from Δ FosB mice treated with either osteogenic or adipogenic agents revealed a significant decrease in adipose cell number and maturation, as opposed to increased expression of genes associated with the osteoblast phenotype (Fig. 4E and data not shown). Thus, *in vivo* and *in vitro* results strongly suggest that Δ FosB overexpression favors commitment of early mesenchymal precursors to the osteoblast lineage, while repressing the differentiation of adipocytes.

[0142] The next and most important question was whether expression of Δ FosB isoforms is biologically significant or whether the observed phenotype merely results from overexpression of a protein that is normally not present in osteoblasts. Using an antibody that recognizes a common epitope in the FosB and Δ FosB isoforms, we found that while FosB levels remained unchanged, Δ FosB levels increased as the cultures differentiated, whereas expression of the further truncated $\Delta 2\Delta$ FosB isoform decreased in the same time frame (Fig. 5A). Thus, stage-specific alternative splicing of *fosB* mRNA and selective initiation site usage of FosB (Chen *et al.*, 1997), rather than expression of FosB itself, regulates osteoblast differentiation.

Example 4. Repression of Adipocyte Differentiation *In Vitro* and *In Vivo*

[0143] Further, the present invention is based on the discovery that Δ FosB overexpression represses adipocyte differentiation. The mechanism by which FosB overexpression might induce differentiation of pluripotent precursor cells toward an osteoblast phenotype at the expense of the adipocytic pathway was then investigated. Using a combination of Northern and Western blot analysis following transient or stable overexpression of Δ FosB or the N-terminally truncated $\Delta 2\Delta$ FosB isoform in primary osteoblasts, or the MC3T3-E1 or C2C12 cell lines, we found that expression of bone matrix proteins and the osteoblast-specific factor OSF2/CBFA1 was upregulated specifically in cells expressing $\Delta 2\Delta$ fosB. Although the $\Delta 2\Delta$ FosB isoform should also be expressed in cells transfected with the full length Δ FosB, usage of the third methionine was not efficient in Δ FosB overexpressing cells, leading to low $\Delta 2\Delta$ FosB expression levels and, interestingly, a parallel decrease or lack of stimulation of osteoblast marker genes (Figs. 5B,C and data not shown) further suggesting that $\Delta 2\Delta$ FosB may be involved in the transgenic phenotype. Surprisingly, expression of $\Delta 2\Delta$ FosB in 3T3-L1 preadipocytes not only downregulated C/EBP mRNA expression even after treatment with adipogenic agents, but caused instead an increase in type I collagen mRNA levels (Dorheim *et al.*, 1993) (Fig. 5D). Furthermore, using gel shift analysis, we observed an

increase in the complexes formed on a consensus AP-1 element using extracts from Δ FosB calvarial cultures. However, in the C2C12-derived stable cell lines, less binding to the same AP-1 element occurred with extracts from Δ FosB overexpressing cells than with extracts from control and $\Delta 2\Delta$ FosB overexpressing cells (Fig. 5E). These findings indicate that the effects of Δ FosB on osteogenesis and adipogenesis are most likely mediated by overexpression of the $\Delta 2\Delta$ FosB isoform. Antibody supershift analysis showed that in primary cultures (data not shown) and in transfected cells (Fig. 5F), the complexes associating with the AP-1 element mainly comprised FosB/ Δ FosB, Fra-2 and JunD, which have been previously implicated in the regulation of osteoblast differentiation (McCabe *et al.*, 1996) as well as Smad4, that has been recently shown to directly associate with AP-1 members (Liberati *et al.*, 1999). Furthermore, this is consistent with previous work, which showed that JunD is the preferred partner for Δ FosB in brain (Chen *et al.*, 1997).

[0144] Thus, $\Delta 2\Delta$ FosB levels are highest early in differentiation and promote expression of osteoblastic markers while repressing adipocytic differentiation. Given that members of the Fos family do not form homodimers, overexpression of Δ FosB isoforms may favor osteoblast commitment at the expense of adipogenesis through the formation of high affinity $\Delta 2\Delta$ FosB heterodimers, predominantly with JunD. Since $\Delta 2\Delta$ FosB is devoid of any known transactivation or repression domains, these complexes could act either as activators of transcription through their Jun partners or prevent the transcriptional activity of other AP-1 complexes by competing for binding to co-activators or to promoter-regulatory elements of genes responsible for commitment to a particular lineage, or both. Interestingly, the promoters of several genes involved in osteoblast (Owen *et al.*, 1990, Banerjee *et al.*, 1996) and adipocytic (Distel *et al.*, 1987, Stephen *et al.*, 1992) differentiation include functional AP-1 binding sites. It was then determined whether Δ FosB and $\Delta 2\Delta$ FosB, which both lack the classical C-terminal transactivation domain of AP-1 family members but still contain the more controversial Fos Homology Domain (FHD), acted primarily as activators or repressors of

transactivation. The stable cell lines were used in transient transactivation assays in order to compare the ability of Δ FosB and $\Delta 2\Delta$ FosB to activate or repress the activity of an osteocalcin (OG2) promoter/CAT reporter gene construct. Transfection of Δ FosB and $\Delta 2\Delta$ FosB with different concentrations of OG2-CAT showed that both Δ FosB- and $\Delta 2\Delta$ FosB-expressing cells induced CAT expression 2- to 8-fold relative to mock-transfected cells, depending on the concentration of reporter plasmid used (data not shown). Thus, both the Δ FosB and the $\Delta 2\Delta$ FosB isoforms are capable of acting as transcriptional activators.

[0145] It has recently been suggested that adipocyte-secreted leptin acts as a negative regulator of bone formation via systemic regulation of osteoblasts by the hypothalamus (Ducy *et al.*, 2000). Although a marked decrease in adipocytes *in vitro* and *in vivo* was observed in transgenic mice, and consequently a decrease in serum leptin ($9.9 \pm 2.4\text{ng/ml}$ vs $3.9 \pm 0.9\text{ng/ml}$, $p < 0.05$), the possibility that the skeletal phenotype observed in Δ FosB transgenic mice resulted from the decreased levels of leptin can be excluded based on the following observations. First, the Δ FosB phenotype is cell autonomous and reproducible *in vitro* even after stable transfection of Δ FosB in the C2C12 cell line, *i.e.*, in the absence of adipocytes. Second, Ducy *et al.* (2000) have shown that the effects of leptin cannot be mimicked *in vitro* and require the presence of the hypothalamus to affect osteoblast function. Third, although leptin is still present in our transgenic mice, the skeletal phenotype of osteosclerosis observed in Δ FosB transgenic mice is much more pronounced than the mild increases in bone formation found in leptin (ob/ob) or leptin receptor (db/db) deficient mice. Finally, of particular interest is the fact that a similar skeletal phenotype is described by Jochum *et al.* in mice overexpressing *fra-1* under the control of the murine H2-K^b class I MHC promoter (submitted jointly). Fra-1 is a closely related Fos family member which, as $\Delta 2\Delta$ FosB, has no known transcriptional activation domain. In this transgenic model, there are no apparent changes in body fat and/or adipocyte differentiation. Thus, the ability of Δ FosB and Fra-1 overexpression to

increase bone formation *in vitro* and *in vivo* is cell autonomous and cannot be accounted for by changes in leptin levels.

[0146] Thus, overexpression of Fos family members which lack transcriptional domains while maintaining their DNA binding and heterodimerization properties, such as $\Delta 2\Delta$ FosB or Fra-1, induce a marked increase in bone formation which leads, *in vivo*, to osteosclerosis. Further understanding of the pathways involved in this anabolic response of the skeleton may help identify new targets for drug development.

Example 5. Method of Determining Whether a Test Agent Induces Bone Formation

[0147] All the transgenic mice are maintained in strict accordance with National Institutes of Health and institutional animal care guidelines. Transgenic mice TetOp- Δ FosB (Chen *et al.*, 1998) are fed with water containing test agent and 5% sucrose. Expression of Δ FosB can be turned off with low doses of doxycycline or induced to very high levels in the absence of doxycycline. Thus, the test agent are administered in the absence and/or in the presence of doxycycline.

[0148] *Luciferase Assay.* Tissues from different organs are obtained by gross dissection. The tissues are homogenized using a sonicator or polytron. The homogenate is centrifuged for 5 min in a microfuge. Ten microliters of the supernatant is used for measurement of luciferase activity in a luminometer by use of the luciferase reporter gene assay kit (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Luciferase activity is normalized to total protein concentration.

[0149] *Western Blotting.* One-dimensional Western blotting for Δ FosB is performed exactly as described by Chen *et al.* (1998), by using an anti-Fos-related antigen antibody (supplied by M. Iadarola, NIH, Bethesda, MD) and chemiluminescence detection (Amersham, Arlington Heights, IL). Levels of Δ FosB immunoreactivity are quantified

by measuring the optical density of specific bands using a Macintosh-based image analysis system with NIH image software.

[0150] Immunohistochemistry. Immunohistochemical analysis of Δ FosB is performed according to Chen *et al.* (1998). Transgenic mice are perfused with 4% paraformaldehyde-phosphate-buffered saline. Sections are labeled with a rabbit polyclonal anti-FosB antibody (1:5000; Santa Cruz Biochemicals, Santa Cruz, CA). Immunoreactivity is detected by diaminobenzidine staining as described by Chen *et al.* (1998).

Sub a² → **[0151] RT-PCR.** Total RNA is isolated from striatum of transgenic mice using the RNAqueous phenol-free total RNA isolation kit (Ambion, Austin, TX) and poly(A)⁺ is isolated using the Oligotex mRNA mini kit (Qiagen, Chatsworth, CA). One microgram of poly(A)⁺ mRNA is used as template for cDNA synthesis using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA). PCR is carried out according to standard protocols from Clontech. PCR primer pairs are designed to distinguish expression of the transgene from the of the endogenous gene. By use of a Δ FosB primer (5' CAG TCT CAG TAC CTG TCT TC 3') and an SV40 primer (5' GTC AGC AGT AGC CTC ATC ATC ACT 3'), it is possible to detect expression of the Δ FosB transgene, which contain both Δ FosB and SV40 sequences.

[0152] Histomorphometric and biochemical analyses as described under Materials and Methods can also be performed on these transgenic mice. Primary murine calvarial cells can also be obtained from the transgenic mice and analyzed as described under Materials and Methods. Other assays that are known to the skilled artisan can also be performed to determine whether the test agent modulates bone formation.

[0153] It should be understood that the foregoing discussion and examples merely present a detailed description of certain preferred embodiments. It therefore should be apparent to those of ordinary skill in the art that various modifications and equivalents can be made without departing from the spirit and scope of the invention. All journal articles, other references, patents, and patent applications that are identified in this patent application are incorporated by reference in their entirety.

09939709.032301

REFERENCES FOR WHICH A COMPLETE CITATION IS NOT PROVIDED IN THE TEXT OF THE SPECIFICATION

- [0154] Anderson, R., Woodbury, D. & Jee, W. Humoral and ionic regulation of osteoclast acidity. *Calcif. Tissue Int.* **39**, 252-258 (1986).
- [0155] Aoki, K., DiDomenico, E., Sims, N. A., *et al.* The tyrosine phosphatase SHP-1 is a negative regulator of osteoclastogenesis and osteoclast resorbing activity: increased resorption and osteopenia in *mev/mev* mutant mice. *Bone* **25**, 261-267 (1999).
- [0156] Arneet, R. & Dempster, D. The effect of pH on bone resorption by rat osteoclasts in vitro. *Endocrinology* **119**, 119-124 (1986).
- [0157] Banerjee, C., Stein, J. L., van Wijnen, A. J., *et al.* TGF- β 1 response in the rat osteocalcin gene is mediated by an AP-1 binding site. *Endocrinology* **137**, 1991-2000 (1996).
- [0158] Baron, R., Vignery, A., Neff, L., Silverglate, A. & Maria, A. S. in *Bone histomorphometry: Technique and interpretation* (ed Recker, R.R.) Vol. 1, 13-35 (CRC Press, Boca Raton, FL, 1983).
- [0159] Bellows, C. G., Heersche, J. N. M. & Aubin, J. E. Determination of the capacity for proliferation and differentiation of osteoprogenitor cells in the presence and absence of dexamethasone. *Developmental Biol.* **140**, 132-138 (1990).
- [0160] Bellows, C. G., Wang, Y. -H., Heersche, J. N. M. & Aubin, J. E. 1,25-dihydroxyvitamin D3 stimulates adipocyte differentiation in cultures of fetal rat calvaria cells: comparison with the effects of dexamethasone. *Endocrinology* **134**, 2221-2229 (1994).
- [0161] Beresford *et al.*, *Journal of Cell Science*, **102**, 341-351 (1992)
- [0162] Cao, Z., Umek, R. M. & McKnight, S. L. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev* **5**, 1538-1552 (1991).
- [0163] Chen, J., Kelz, M. B., Zeng, G., *et al.* Transgenic animals with inducible, targeted gene expression in brain. *Mol. Pharmacol.* **54**, 495-503 (1998).

- [0164] Chen, J., Kelz, M. B., Hope, B. T., Nakabeppu, Y. & Nestler, E. J. Chronic Fos-related antigens: stable variants of Δ FosB induced in brain by chronic treatments. *J Neurosci* **17**, 4933-4941 (1997).
- [0165] Distel, R. J., Ro, H. S., Rosen, B. S., Groves, D. L. & Spiegelman, B. M. Nucleoprotein complexes that regulate gene expression in adipocyte differentiation: direct participation of *c-fos*. *Cell* **49**, 835-844 (1987).
- [0166] Dorheim, M. A., Sullivan, M., Dandapani, V., *et al.* Osteoblastic gene expression during adipogenesis in hematopoietic supporting murine bone marrow stromal cells. *J Cell Physiol* **154**, 317-328 (1993).
- [0167] Ducy, P., Desbois, C., Boyce, B., *et al.* Increased bone formation in osteocalcin-deficient mice. *Nature* **382**, 448-452 (1996).
- [0168] Ducy, P., Amling, M., Takeda, S., *et al.* Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell* **100**, 197-207 (2000).
- [0169] Franzoso, G., Carlson, L., Xing, L., *et al.* Requirement for NF- κ B in osteoclast and B-cell development. *Genes & Dev.* **11**, 3482-3496 (1997).
- [0170] Gimble *et al.*, *Journal of Cellular Biochemistry*, **58**, 393-402 (1995)
- [0171] Grigoriadis, A. E., Shellander, K., Wang, Z-Q. & Wagner, E. F. Osteoblasts are target cells for transformation in *c-fos* transgenic mice. *J. Cell Biol.* **122**, 685-701 (1993).
- [0172] Gruda, M. C., van Amsterdam, J., Rizzo, C. A., Durham, S. K., Lira, S. & Bravo, R. Expression of FosB during mouse development: normal development of FosB knockout mice. *Oncogene* **12**, 2177-2185 (1996).
- [0173] Hayman, A. R., Jones, S. J., Boyde, A., *et al.* Mice lacking tartrate-resistant acid phosphatase (Acp 5) have disrupted endochondral ossification and mild osteopetrosis. *Development* **122**, 3151-3162 (1996).
- [0174] Johnson, R. S., Spiegelman, B. M. & Papaioannou, V. Pleiotropic effects of a null mutation in the *c-fos* proto-oncogene. *Cell* **71**, 577-586 (1992).
- [0175] Kelz, M. B., Chen, J., Carlezon, Q. A., *et al.* Expression of the transcription factor Δ FosB in the brain controls sensitivity to cocaine. *Nature* **401**, 272-276 (1999).

- [0176] Liberati, N., Datto, M. B., Frederick, J. P., *et al.* Smads bind directly to the Jun family of AP-1 transcription factors. *Proc. Natl. Acad. Sci. USA* **96**, 4844-4849 (1999).
- [0177] Marks, S. C., Jr. & Lane, P. W. Osteopetrosis, a new recessive skeletal mutation on chromosome 12 of the mouse. *J. Heredity* **67**, 11-18 (1976).
- [0178] McCabe, L. R., Banerjee, C., Kundu, R., *et al.* Developmental expression and activities of specific Fos and Jun proteins are functionally related to osteoblast maturation: Role of Fra-2 and Jun D during differentiation. *Endocrinology* **137**, 4398-4408 (1996).
- [0179] Nakabeppu, Y. & Nathans, D. A naturally occurring truncated form of FosB that inhibits Fos/Jun transcriptional activity. *Cell* **64**, 751-759 (1991).
- [0180] Nestler *et al.*, *Brain Research*, **835**, 10-17 (1999)
- [0181] Owen, T. A., Bortell, R., Yocum, S. A., *et al.* Coordinate occupancy of AP-1 sites in the vitamin D-responsive and CCAAT box elements by Fos-Jun in the osteocalcin gene: Model for phenotype suppression of transcription. *Proc Natl Acad Sci U S A* **87**, 9990-9994 (1990).
- [0182] Parfitt, A. M., Drezner, M. K., Glorieux, F. H., *et al.* Bone histomorphometry: Standardization of nomenclature, symbols, and units. Report of the ASBMR histomorphometry nomenclature committee. *J Bone Miner Res* **2**, 595-610 (1987).
- [0183] Quarles, L. D., Yohay, D. A., Lever, L. W., Caton, R. & Wenstrup, R. J. Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an *in vitro* model of osteoblast development. *J. Bone Miner. Res.* **7**, 683-692 (1992).
- [0184] Sabatakos, G., Davies, G. E., Grosse, M., Cryer, A. & Ramji, D. P. Expression of the genes encoding CCAAT-enhancer binding protein isoforms in the mouse mammary gland during lactation and involution. *Biochem J* **334**, 205-210 (1998).
- [0185] Sambrook, J. Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, New York, 1989).

- [0186] Schreiber, E., Matthias, P., Muller, M. M. & Schaffner, W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* **17**, 6419 (1989).
- [0187] Seifert, M. F. & Marks, S. C. Morphological evidence of reduced bone resorption in the osteosclerotic (*oc*) mouse. *Am J Anat* **172**, 141-153 (1985).
- [0188] Simonet, W. S., Lacey, D. L., Dunstan, C. R., *et al.* Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. *Cell* **89**, 309-319 (1997).
- [0189] Soriano, P., Montgomery, C., Geske, R. & Bradley, A. Targeted disruption of the *c-src* proto-oncogene leads to osteopetrosis in mice. *Cell* **64**, 693-702 (1991).
- [0190] Stephens, J. M., Butts, M. D. & Pekala, P. H. Regulation of transcription factor mRNA accumulation during 3T3-L1 preadipocyte differentiation by tumour necrosis factor-alpha. *J. Mol. Endocrinol.* **9**, 61-72 (1992).
- [0191] Takahashi, N., Yamana, H., Yoshiki, S., *et al.* Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. *Endocrinology* **122**, 1373-1382 (1988).
- [0192] Tondravi, M. M., McKercher, S. R., Anderson, K., *et al.* Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. *Nature* **386**, 81-84 (1997).
- [0193] Wang, Z-Q., Liang, J., Schellander, K., Wagner, E. F. & Grigoriadis, A. E. *c-fos*-induced osteosarcoma formation in transgenic mice: cooperativity with *c-jun* and the role of endogenous *c-fos*. *Cancer Research* **55**, 6244-6251 (1995).
- [0194] Wang, Z. -Q., Ovitt, C., Grigoriadis, A. E., Mohle-Steinlein, U., Ruther, U. & Wagner, E. F. Bone and haematopoietic defects in mice lacking *c-fos*. *Nature* **360**, 741-745 (1992).